Psoriasis Upregulated Phorbolin-1 Shares Structural but not Functional Similarity to the mRNA-Editing Protein Apobec-1

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Earlier studies of psoriatic and normal primary keratinocytes treated with phorbol 12-myristate-1-acetate identified two low-molecular-weight proteins, termed phorbolin-1 (20 kDa; pI 6.6) and phorbolin-2 (17.6 kDa; pI 6.5). As a first step towards elucidating the role of these proteins in psoriasis, we report here the molecular cloning and chromosomal mapping of phorbolin-1 and a related cDNA that codes for a protein exhibiting a similar amino acid sequence. The phorbolins were mapped to position 22q13 immediately centromeric to the c-*sis* proto-oncogene. Transient expression of the phorbolin-1 cDNA in COS cells and by *in vitro* transcription/translation, yielded polypeptides that comigrated with phorbolins-1 and -2. Comparative sequence analysis revealed 22% overall identity and a similarity of 44% of the phorbolins to apobec-1, the catalytic subunit of the mammalian apolipoprotein B mRNA editing enzyme; however, recombinant-expressed phorbolin-1 exhibited no cytidine deaminase activity, using either a monomeric nucleoside or apolipoprotein B cRNA as substrate, and failed to bind an AU-rich RNA template. Whereas the precise function of the phorbolins remains to be elucidated, the current data suggest that it is unlikely to include a role in the post-transcriptional modification of RNA in a manner analogous to that described for apobec-1. *Key words: cDNA cloning/mRNA editing.*

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Psoriasis is a hyperproliferative inflammatory disease that affects about 2% of the population in northern Europe and Scandinavia. The disease is histopathologically characterized by hyperproliferation and aberrant differentiation of the epidermal keratinocytes and by invasion of neutrophils and T lymphocytes (Wright and Camplejohn, 1985). Currently, relatively little is known about the molecular mechanisms underlying the disease, although numerous studies have been devoted to the analysis of gene products known to be involved in proliferation and immune activation. These include: cytokines, growth factors, adhesion molecules, proteases, proto-oncogenes, as well as other proteins of yet unknown functions (Elder *et al*, 1990; Madsen *et al*, 1991, 1992; Wiedow *et al*, 1993; Barker, 1994, 1995; Valdimarsson *et al*, 1995; Christophers, 1996; Norris *et al*, 1997). Recently, we presented evidence suggesting that the protein kinase C (PKC) signaling pathway may be altered in psoriasis, as treatment of normal keratinocytes with phorbol 12-myristate 13-acetate (PMA), an activator of PKC, leads to the overexpression of proteins, including phorbolins-1 and -2, that are also highly expressed in psoriatic lesions (Rasmussen and Celis, 1993). The effect was not elicited by other effectors such as second messengers (Bt_2cAMP, Bt_2cGMP) and cytokines [basic fibroblast growth factor, transforming growth factor-α, tumor necrosis factors-α and -β, interleukin (IL) -1α, IL-1β, IL-2, IL-3, IL-6, IL-7, IL-8, and interferons α and γ], but was partly mimicked by fetal bovine serum, which is known to induce abnormal differentiation, a characteristic of psoriatic keratinocytes (Rasmussen and Celis, 1993). Evidence for altered levels of PKC in psoriasis has been reported by several laboratories (Horn *et al*, 1987; Fisher *et al*, 1990, 1993; Venneker *et al*, 1994), and it has been suggested that PKC inhibitors may have a therapeutic value (Gupta *et al*, 1988; Hegemann *et al*, 1992a, b).

In this study, we report the cloning and expression of phorbolin-1 and of a homologous cDNA called phorbolin-1-related cDNA. Comparative cDNA sequence analysis revealed 20% identity between the phorbolins-1 and -2 and apobec-1, the catalytic subunit of the mammalian apolipoprotein B mRNA editing enzyme complex (Teng *et al*, 1993). In humans, apobec-1 is expressed in the small intestine, where it functions in the post-transcriptional deamination of C to U of apolipoprotein B mRNA (Teng *et al*, 1993), a process referred to as RNA editing (Ashkenas, 1997). The conversion of a CAA codon (encoding glutamine) to a UAA stop codon in the primary nuclear apolipoprotein B transcript results in the tissue-specific production of apoB48 (Teng *et al*, 1993). One of the regions homologous between phorbolin-1 and apobec-1

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Abbreviations: SSP, sample spot number; uORF, upstream open-reading frames.

was one that encompassed the zinc-coordinating residues corresponding to the active site of known cytidine deaminases (Bhattacharya *et al*, 1994). This apparent similarity in primary sequences led us to determine directly whether phorbolin-1 exhibits activity as a cytidine deaminase. Our results show that, despite the apparent homology, the phorbolin-1 protein demonstrates no detectable cytidine deaminase activity when assayed either on a monomeric nucleoside substrate or a synthetic apoB RNA template. Accordingly, the precise function of the phorbolins remains elusive. The possibility, however, that these proteins may play a part in the editing of other mRNA through an as yet unknown mechanism cannot be excluded at present.

MATERIALS AND METHODS

Normal primary human keratinocytes Strips of normal skin were washed three times in Hank's buffered saline (HBSS) and placed in 0.25% trypsin in HBSS at 4°C for at least 24 h. Following incubation, the strips were washed three times in serum-free keratinocyte medium (Gibco, Cleveland, OH) containing antibiotics (100 U penicillin per ml; 50 μg streptomycin per ml; Biochrom KG, Germany; 5 ng epidermal growth factor per ml and 50 μg bovine pituitary extract per ml). The epidermis was then detached from the dermis and resuspended in the same medium. Keratinocytes were detached by vigorous shaking, washed two to three times in serum-free keratinocyte medium and plated in 35 mm culture dishes (coated with human dermal extract) that contained 3 ml of complete serum-free keratinocyte medium. Keratinocytes were fed with fresh medium every day and loosely attached cells were eliminated by pipetting the medium up and down with the aid of an automatic pipette. Cell were labeled for 20 h in serum-free keratinocyte medium lacking methionine and containing 50 μCi of [^{35}S]methionine (SJ 204 Amersham, Uppsala, Sweden) per 0.1 ml of medium. At the end of the labeling period the cells were resuspended in lysis solution (Bravo and Celis, 1982).

Preparation of human dermal extracts Strips of normal skin washed in HBSS were placed in HBSS containing 1 M NaCl for 72 h at 4°C. The epidermis was then peeled off and the dermis washed twice in HBSS. Thereafter, the dermis was homogenized in the same buffer and the soluble extract was recovered by centrifugation. The extract was then dialyzed against HBSS, filter sterilized, and stored at 4°C. Petri dishes were coated with the extract overnight at 4°C (20 μg per ml). The extract was then aspirated and the plates were kept at 4°C until use.

Production of antibodies in rabbits A polymerase chain reaction fragment encoding the 103 C-terminal amino acids of phorbolin-1 was cloned into pT7-PL vector (Christensen *et al*, 1991) and expressed as a hexaHis fusion protein in *Escherichia coli* BL21(DE3) by isopropyl-β-D-thiogalactopyranoside induction. The protein was purified under denaturing conditions on a Ni^{2+} column (Hochuli *et al*, 1987), and thereafter exchanged by gel filtration into 150 mM KCl and 20 mM HEPES pH 7.4. The purified protein was mixed with Freund's complete adjuvant and injected into a rabbit, followed by a boost injection using incomplete Freund's 4 wk later. Blood was collected after additional 8 d and the specificity of the antibody was determined by two-dimensional (2D) immunoblotting of PMA-treated keratinocytes. The serum was precipitated with an equal volume of saturated $(NH_4)_2SO_4$ and the resulting precipitate was dialyzed against HBSS. Aliquots were kept frozen at –80°C.

***In vitro* transcription/translation** The TNT Coupled Reticulocyte Lysate system from Promega (Madison, WI) was used according to the manufacturer's description. The phorbolin-1 and phorbolin-1-related cDNAs were inserted into pBluescript-SK (derived from the λZAP library) and expressed using T3 polymerase. The phorbolin-1-related cDNA was transcribed from position 181. Following translation in the presence of [^{35}S]methionine (Madsen *et al*, 1995), the lysates were mixed with an equal volume of lysis solution and subjected to 2D polyacrylamide gel electrophoresis (PAGE), described below (Bravo and Celis, 1982).

RNA editing, RNA binding, and PHYLIP analysis A recombinant form of apobec-1, the catalytic subunit of the mammalian apolipoproteinB mRNA editing enzyme, was expressed as a glutathione S-transferase (GST)-fusion protein as previously described (Anant *et al*, 1995; MacGinnitie *et al*, 1995). Recombinant apobec-1 as well as *in vitro* transcription/translation synthesized apobec-1 was used in an *in vitro* editing assay with a synthetic apolipoprotein B RNA template containing 470 nt spanning the edited cytidine at position 6666 in the apolipoproteinB cDNA (Anant *et al*, 1995; MacGinnitie *et al*, 1995). Twenty femtomol of RNA was incubated with 500 ng of GST-apobec-1 in the presence of 10 μg of chicken intestinal S100 extracts (an obligate source of auxiliary factors for *in vitro* RNA editing) and the reaction allowed to proceed for 2 h at 30°C. In a parallel set of incubations, 2 μl of the *in vitro* transcription/translation products of phorbolin-1 was used along with 10 μg chicken S100 extract. The reaction products were used to determine the extent to which the cytidine at position 6666 is converted to uridine by means of a primer extension/dideoxy chain termination reaction. Specifically, a 35-mer oligonucleotide complementary to the sense strand of apolipoproteinB mRNA in the region starting 8 nt downstream of the edited base, was annealed and allowed to extend with reverse transcriptase in the presence of μM dATP, dCTP, dTTP, and high concentrations of ddGTP (500 μM) as a chain terminator. If the cytidine at position 6666 is unedited, the reaction terminates at this position, giving an extension product of 43 nt. If nt 6666 is edited to a uridine, the extension continues to the next upstream cytidine, located at position 6655, which results in an extension product of 54 nt. The extended products are resolved on an 8% urea–polyacrylamide gel.

Ultraviolet cross-linking was conducted using a [^{32}P]-labeled RNA template containing 105 nt of rat apolipoproteinB mRNA spanning the edited base (Giannoni *et al*, 1994; MacGinnitie *et al*, 1995). Approximately 50 000 cpm was incubated with recombinant GST-apobec-1 or GST-phorbolin-1-related protein for 20 min at room temperature and the products treated with RNase T1 and heparin prior to ultraviolet cross-linking in a Strategene cross-linker set at 250 mJ per cm^2. The reaction products were then subjected to reducing 10% sodium dodecyl sulfate (SDS)–PAGE and fluorography.

Phylogenetic analysis was performed by PHYLIP ver. 3.572c using the active site of cytosine nucleotide and nucleoside deaminases and the RNA editing enzymes (Felsenstein, 1988) The distance matrix between the various proteins was calculated by PROTDIST program using the Dayhoff PAM matrix method. The matrix was used to generate a tree by the KITCH program using the Fitch-Margoliash method and the tree plotted using the Drawgram program.

2D PAGE SDS–PAGE, 15% running gel; 5% stacking gel) was carried out essentially as described by Laemmli (1970). 2D gel electrophoresis (isoelectric focusing, nonequilibrium pH gradient electrophoresis) (O'Farrell, 1975) was carried out as described by Celis *et al* (1994b). In short, the first dimension was performed either as isoelectric focusing (18 h at 400 V) in 125 × 2 mm 4% wt/vol polyacrylamide gels containing 2% wt/vol carrier ampholytes (1.6% pH 5–7, Serva, Heidelberg, Germany; 0.4% pH 3.5–10, Pharmacia Biosystems AS, Uppsala, Sweden). First-dimensional gels placed in 3 cm Petri dishes were equilibrated (3 min at room temperature) in 3.5 ml of equilibration solution (0.06 M Tris–HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, and 10% glycerol) (O'Farrell, 1975). Gels were then stored at –20°C until use (usually 1–2 wk). First-dimensional gels were applied to the second dimension with the aid of agarose solution (0.06 M Tris–HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol, 1% agarose, and 0.002% Bromophenol Blue) (O'Farrell, 1975). Gels were run at room temperature at 10 mA for 4 h and at 3 mA overnight. Solutions and procedures used to run 2D gels in our laboratory are described in detail at: http://biobase.dk/cgi-bin/celis

Microsequencing Protein spots from several Coomassie-stained, dried 2D gels were cut and combined and concentrated by elution-concentration gel electrophoresis (Rasmussen *et al*, 1991; Vanderkerckhove and Rasmussen, 1994). Polyvinylidene difluoride bound proteins were visualized by staining with 0.1% Amido Black in 45.5% methanol, 9% acetic acid and destained in water. The wet protein bands were cut out and placed in 500 μl 0.2% polyvinylpyrolidone in water for 15 min in order to quench remaining protein binding sites on the membrane. After washing the membrane was placed in 100 μl digestion buffer containing 1 μg porcine trypsin. The digestion proceeded for 4 h at 37°C. The tryptic peptides were released from the membrane by adding 10 μl trifluoracetic acid (TFA) and separated by reversed-phase high-performance liquid chromatography on a C18 C4 reversed-phase column (0.46 × 25 cm; Vydac Separations Group). The major peptides were further dried in a SpeedVac (Savant) concentrator and stored at –20°C prior to sequence analysis. Peptides were selected for amino acid sequence, redissolved in 30 μl of 0.1% TFA/30% acetonitrile before loading on a precycled Polybrene-coated glass filter. The sequence analysis was carried out with pulse-liquid sequencer (model 477A, Applied Biosystems, Forster City, CA) and run with the sequencing program recommended by the manufacturer.

2D gel immunoblotting 2D gels for immunoblotting were placed for 5 min in TGM (Tris, glycine, methanol) prior to transfer (24 h at 130 mA)

(Towbin *et al*, 1979; Celis *et al*, 1994a). Following protein transfer (total cellular extracts from keratinocytes mixed with small amounts of [^{35}S]methionine-labeled extracts) on to nitrocellulose, the sheet or strip was dried and exposed to an X-ray film. The amount of keratinocyte cell extract applied to the gels was carefully calibrated to obtain the maximum amount of protein transferred without affecting the quality of the 2D gels. Dry sheets could be kept for extended periods of time (up to 3 mo) without significant change in the reactivity of the proteins. For immunodetection of the antigen the following procedure was used: nitrocellulose strips or sheets were incubated for 16 h at room temperature in HBSS containing 1.5% bovine hemoglobin (Sigma, St. Louis, MO, type II). The blots were then incubated at room temperature for 2 h with HBSS containing 2% hemoglobin and a 1:100 of the first antibody. After three washes with HBSS (3 × 30 min), the blot was incubated with peroxidase-labeled second antibody (Dakopatts AS, Denmark; 1 : 100 dilution) for 2 h. It was then washed three times (3 × 30 min) with HBSS and finally developed in enhanced chemiluminescence solution (Amersham, Uppsala, Sweden), and exposed to X-ray film.

Chromosome localization Fluorescence *in situ* hybridization mapping was carried out by nick labeling of the phorbolin-1 cDNA in the presence of bio-dUTP followed by hybridization to human metaphase chromosomes. Metaphases were examined in Zeiss Periplan epifluorescence microscope (Tommerup and Vissing, 1995). The phorbolin cDNA probes were hybridized to high-density cosmid filters prepared from the libraries and a unique set of 26 positive cosmid clones was identified. Random hexamer labeled *Hin*dIII digested cosmid DNA was denatured in the presence of 2.5 mg sonicated human placental DNA per ml (Sigma). This was hybridized overnight at 65°C to a single high-density gridded chromosome 22 YAC filter (Collins *et al*, 1995). The filter was washed with 0.5 × sodium citrate/chloride buffer at 65°C for 1 h and visualized by autoradiography. The cosmid probe was positioned relative to markers in the chromosome 22 YAC map by YAC marker content (Collins *et al*, 1995).

Purification of RNA, preparation, and screening of cDNA libraries Total RNA was purified from trypsin detached psoriatic epidermis by homogenization in a 4 M solution of guanidinium thiocyanate and 0.1 M 2-mercaptoethanol, followed by sedimentation through cesium chloride (Chirgwin *et al*, 1979). Messenger RNA was isolated using a oligo-dT column from Stratagene (La Jolla, CA). The cDNA library was constructed by priming mRNA with $(dT)_{15}$ containing *Xho*I and *Sac*I site at the 5′ end. The cDNA was ligated into λ Uni-ZAP-XR cut with *Xho*I and *Eco*RI (Gubler and Hoffman, 1983). The amplified library was plated on 22 × 22 cm plates containing 1.5 × 10^5 plaque-forming units, and overlaid with nylon filters (Hybond-N, Amersham). The DNA was covalently linked to the filters by ultraviolet light. Following 5′ labeling the degenerated oligonucleotides were used to screen replica filters using a modification of the tetramethylammonium chloride salt method (Honoré and Madsen, 1997). The filters were sealed in plastic bags, and autoradiography was done with Kodak X-ray films. Positive clones were purified by repeated platings and rescued in pBluescript. The purified clones were sequenced by the dideoxy method (Sanger *et al*, 1977).

RESULTS

Microsequencing, molecular cloning, and expression of phorbolin-1 Coommassie brilliant blue-stained phorbolin-1 recovered from several isoelectric focusing 2D gels from psoriatic keratinocytes were pooled, and tryptic peptides were sequenced (**Fig 1**). A cDNA that coded for phorbolin-1 was cloned by screening a λZAP psoriatic epidermis cDNA expression library using degenerate oligodeoxyribonucleotides back-translated from one of the peptide sequences (IYDYDPLYK). Several positive cDNA clones were isolated and sequenced, but only the deduced amino acid sequence from clone 4359 contained all of the tryptic peptides that were microsequenced (**Fig 1**). As shown in **Fig 1**, the open-reading frame of clone 4359 encodes a protein comprising 199 amino acids, with a molecular mass of 23.012 kDa and a calculated pI of 6.83. These values are very close to those recorded for phorbolin-1 in the 2D PAGE database of keratinocyte proteins (20.0 kDa; pI 6.6) (Celis *et al*, 1996). (The 2D PAGE human keratinocyte protein database is also available on the internet: http//biobase.dk/cgi-bin/celis) The 3′-UTR region of clone 4359 contains an ALU repeat sequence spanning positions 841–1130 (**Fig 1**). To substantiate further that clone 4359 codes for phorbolin-1, we compared the 2D PAGE profile of clone 4359 overexpressed

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   1  GGATCTTAACACCACGCCTTGAGCAAGTCGCAAGAGCGGGAGGACACAGACCAGGAACCG

   1                M  E  A  S  P  A  S  G  P  R  H  L  M  D  P
  61  AGAAGGGACAAGCACATGGAAGCCAGCCCAGCATCCGGGCCCAGACACTTGATGGATCCA
                  *** 76                                  *** 112
  16  H  I  F  T  S  N  F  N  N  G  I  G  R  H  K  T  Y  L  C  Y
 121  CACATATTCACTTCCAACTTTAACAATGGCATTGGAAGGCATAAGACCTACCTGTGCTAC

  36  E  V  E  R  L  D  N  G  T  S  V  K  M  D  Q  H  R  G  F  L
 181  GAAGTGGAGCGCCTGGACAATGGCACCTCGGTCAAGATGGACCAGCACAGGGGCTTTCTA
                                          *** 217
  56  H  N  Q  A  K  N  L  L  C  G  F  Y  G  R  H  A  E  L  R  F
 241  CACAACCAGGCTAAGAATCTTCTCTGTGGCTTTTACGGCCGCCATGCGGAGCTGCGCTTC

  76  L  D  L  V  P  S  L  Q  L  D  P  A  Q  I  Y  R  V  T  W  F
 301  TTGGACCTGGTTCCTTCTTTGCAGTTGGACCCGGCCCAGATCTACAGGGTCACTTGGTTC

  96  I  S  W  S  P  C  F  S  W  G  C  A  G  E  V  R  A  F  L  Q
 361  ATCTCCTGGAGCCCCTGCTTCTCCTGGGGCTGTGCCGGGGAAGTGCGTGCGTTCCTTCAG

 116  E  N  T  H  V  R  L  R  I  F  A  A  R  I  Y  D  Y  D  P  L
 421  GAGAACACACACGTGAGACTGCGCATCTTCGCTGCCCGCATCTATGATTACGACCCCCTA

 136  Y  K  E  A  L  Q  M  L  R  D  A  G  A  Q  V  S  I  M  T  Y
 481  TATAAGGAGGCGCTGCAAATGCTGCGGGATGCTGGGGCCCAAGTCTCCATCATGACCTAC

 156  D  E  F  K  H  C  W  D  T  F  V  D  H  Q  G  C  P  F  Q  P
 541  GATGAATTTAAGCACTGCTGGGACACCTTTGTGGACCACCAGGGATGTCCCTTCCAGCCC

 176  W  D  G  L  D  E  H  S  Q  A  L  S  G  R  L  R  A  I  L  Q
 601  TGGGATGGACTAGATGAGCACAGCCAAGCCCTGAGTGGGAGGCTGCGGGCCATTCTCCAG

 196  N  Q  G  N  stop
 661  AATCAGGGAAACTGAAGGATGGGCCTCAGTCTCTAAGGAAGGCAGAGACCTGGGTTGAGC
 721  AGCAGAATAAAAGATCTTCTTCCAAGAAATGCAAACAGACCGTTCACCACCATCTCCAGC
 781  TGCTCACAGACGCCAGCAAAGCAGTATGCTCCCGATCAAGTAGATTTTTAAAAAATCAGA
 841  GTGGGCCGGGCGCGGCTGCACGCCTGTAATCCCAGCACTTTGGAGGCCAAGGCGGGTGGA
 901  TCACGAGGTCAGGAGATCGAGACCATCCTGGCTAACACGGTGAAACCCTGTCTCTACTAA
 961  AAATACAAAAAATTAGCCAGGGGTGGTGCGGGCGCCTGTAGTCCCAGCTACTCTGGAGGC
1021  TGAGGCAGGAGAGTACGTGAACCCGGGAGGCAGAGCTTGCGGTGAGCCGAGATTGCGCTA
1081  CTGCACTCCAGCCTGGGCGACAGNACCAGACTCCATCTCAAAAAAAAAAAAAAACCAGACT
1141  GAATTAATTTTAACTGAAAATTTCTCTTATGTTCCAAGGTACACAATAGGTAAGATTATG
1201  CTCAATATTCTCAGAATAATTTTCAATGTATTAATGAAATGAAATGATAATTTGGCTTCA
1261  TATCTAGACTAACACAAAATTAAGAATCTTCCATAATTGCTTTTGCTCAGTAACTGTGTC
1321  ATGAATTGCAAGAGTTTCCACAAACACT polyA 1348
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Figure 1. Nucleotide and deduced amino acid sequence of the phorbolin-1 cDNA. Nucleotide and corresponding amino acid sequence of the cDNA clone encoding for isoelectric focusing SSP 2116 (Rasmussen and Celis, 1993). The cDNA, which contains 1348 bp, codes for a protein having 199 amino acids starting at nucleotide position 76 and ending with a termination codon at position 675. Potential AUG start codons are marked with asterisks, and partial peptide sequences obtained by microsequencing are underlined. Accession number U03891.

in COS-1 cells with that of the native protein overexpressed in PMA-treated keratinocytes (**Fig 2*A, B***). By superimposing the autoradiograms it was possible to show that the protein overexpressed by COS-1 cells transfected with clone 4359 comigrated with phorbolin-1 expressed by PMA-treated keratinocytes (**Fig 2*A, B***). Taken together, the results provide strong evidence that clone 4359 codes for phorbolin-1.

COS-1 cells transfected with clone 4359 express, in addition to phorbolin-1, a low-molecular-weight protein that comigrates with phorbolin-2 (**Fig 2*A, B***). Both this protein and phorbolin-1 reacted with a rabbit polyclonal antiserum directed against the 103 hexaHis-tagged C-terminal amino acids of clone 4359 expressed in bacteria (**Fig 2*C***), suggesting that the low-molecular-weight protein is derived from clone 4359. Non-transfected COS-1 cells did not react with the anti-serum (not shown). The increased sensitivity of the enhanced chemiluminescence detection procedure revealed, in addition, acidic variants of both phorbolin proteins (**Fig 2*D***). The nature of the acidic variants is at present not known. 2D PAGE analysis of proteins expressed by clone 4359 using the TNT-coupled *in vitro* transcription/translation system yielded essentially the same results (**Fig 2*D***) as those observed in the COS-1 transfected cells (**Fig 2*A, C***). At present, we do not know whether phorbolin-2 is derived as a result of differential AUG codon usage or of proteolysis of phorbolin-1. Analysis of the phorbolin-1 cDNA sequence revealed three AUG codons located downstream of an in-frame stop codon at position 20 that could potentially be used as translational initiation codons (**Fig 1**). Differential usage of AUG codons may explain the different ratios between phorbolin-1 and 2 observed in **Fig 2**(***A, C, D***), as ribosome scanning of the 5′ mRNA may select AUG codons with different probabilities in the various expression systems analyzed.

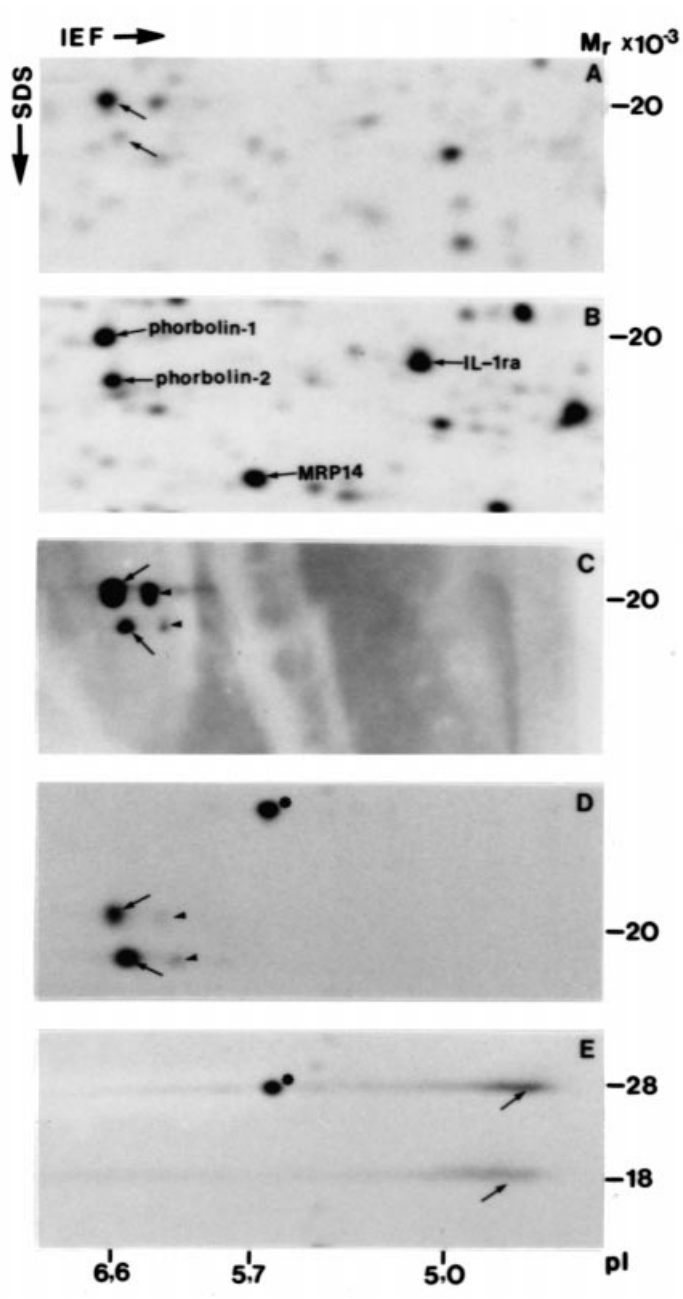

Figure 2. Expression of clone 4359. (*A*) 2D PAGE autoradiogram of [^{35}S]methionine-labeled polypeptides from COS-1 cells transfected with clone 4359, inserted into the pMT21 eukaryotic expression vector. Overexpressed proteins are indicated by *arrows*, these proteins are not detected in non-transfected COS-1 cells (not shown). (*B*) 2D PAGE autoradiogram of [^{35}S]methionine-labeled polypeptides from primary human keratinocytes treated for 20 h with PMA (100 ng per ml), 5 d after plating. PMA-induced proteins are indicated by name. (*C*) 2D immunoblot of COS-1 cells transfected with clone 4359, using a rabbit anti-serum directed against the C-terminal of clone 4359. (*D*) 2D PAGE autoradiogram of clone 4359 assayed in the *in vitro* transcription/translation system. (*E*) 2D PAGE autoradiogram of the phorbolin-1-related cDNA expressed by the *in vitro* transcription/translation system. Overexpressed proteins in *C–E* are indicated by *arrows*, acidic variants are indicated by *arrowheads*. Asterisks in *D and E* indicates a common translational product that is not related to the expressed cDNA, as this protein also is detected when expressing unrelated cDNA (Madsen *et al*, 1995).

Phorbolin-1-related cDNA Sequence analysis of several clones hybridizing to the fully degenerate oligodeoxyribonucleotide probes revealed cDNA clones closely related to the phorbolin-1 cDNA. The deduced amino acid sequence exhibited an overall identity of

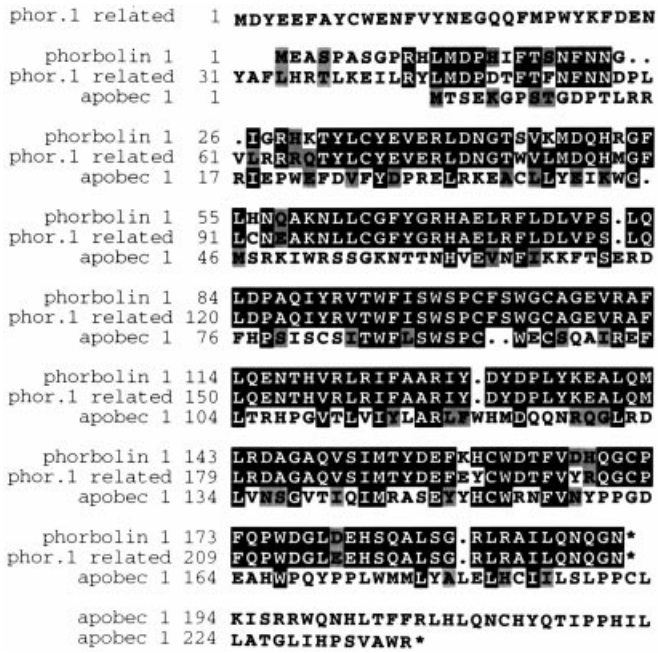

Figure 3. Alignment of the phorbolins and apobec-1. Identical residues are highlighted in a black background whereas similar residues are in gray. The region containing the active site and zinc coordinating spans residues 61–96 in the apobec-1 amino acid sequence.

89% to the phorbolin-1 amino acid sequence, with the major differences found near the amino terminus (**Fig 3**). The mRNA for this cDNA has many AUG codons upstream of an in-frame stop codon at position 372 and several potential start codons downstream of this stop codon (**Fig 4**). Analysis of the *in vitro* transcription/translation products from the phorbolin-1-related cDNA clone B, which starts at position 181 (and therefore has no interference from the AUG codons upstream of this position) revealed two proteins having pI of approximately 5 and apparent molecular masses of 28 and 18 kDa, respectively. From the differences in molecular weights and pI, we conclude that the phorbolin-1-related cDNA encodes proteins that are unrelated to phorbolin-1 and -2 (**Fig 2*F***). Sequence analysis of seven independent phorbolin-1-related cDNA clones revealed sequence variation at position 673, which in turn creates further uORF (**Fig 4**). In three of the clones there is a T at position 673, creating an in-frame UGA stop codon, whereas in four clones there is a C at that position, thereby coding for an arginine (CGA) at position 63 (**Fig 4**). Sequence analysis of the corresponding genomic positions of two independent cosmid clones showed that a C is indeed present. Further analysis of more alleles, however, is necessary in order to establish whether genomic variation exists at this position.

Protein alignment and functional assay The predicted amino acid sequence derived from the phorbolin cDNA clones was used to search the GenBank, using the Blast Search program to identify homologous sequences. The search revealed that phorbolin-1 had a significant homology to apobec-1, with an overall identity of 20% and a similarity of 44%, requiring six gaps to be inserted to make them align (**Fig 3**). In particular, the homologous regions in the phorbolins include the sequence His-(Ala)-Glu-X-X-Phe-X-(19)-Phe-(Ile)-Ser-Trp-Ser-Pro-Cys-X-(4)-Cys, which are amino acids 70–106 in the phorbolins (**Fig 3**). In apobec-1, this region has been shown to encompass the active site of the enzyme and contains the zinc coordinating residues (one histidine and two cystine residues) required for cytidine deaminase activity, as well as the conserved glutamate residue that functions as a proton donor (Anant *et al*, 1995; MacGinnitie *et al*, 1995). In addition, the proline (at position 92 in apobec-1) and the two phenylalanine residues (at positions 66 and 87 in apobec-1) are conserved in the phorbolins.

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   1  CAGGGACAAGCGTATCTAAGAGGCTGAACATGAATCCACAGATCAGAAATCCGATGGAG
  60  CGGATGTATCGAGACACATTCTACGACAACTTTGAAAACGAACCCATCCTCTATGGTCGG
 120  AGCTACACTTGGCTGTGCTATGAAGTGAAAATAAAGAGGGGCCGCTCAAATCTCCTTTGG
 180  GACACAGGGGTCTTTCGAGGCCAGGTGTATTTCGAGCCAGGTGTATTTCGAGCCTCAGTA
 240  CCACGCAGAAATGTGCTTCCTCTCTTGGTTCTGTGGCAACCAGCTGCCTGCTTACAAGTG
 300  TTTCCAGATCACCTGGTTTGTATCCTGGACCCCCTGCCCGGACTGTGTGGCGAACGTGGC
 360  CGAATTCCTGTCTGAGCACCCCAATGTCACCCTCACCATCTCCGCCGCCCGCCTCTACTA
 420  CTACTGGGAAAGAGATTACCGAAGGCGCTCTGCAGGCTGAGTCAGGCAGGAGCCCGCGTG
   1        M  D  Y  E  E  F  A  Y  C  W  E  N  F  V  Y  N  E  G
 480  AAGATCATGGACTATGAAGAATTTGCATACTGCTGGGAAAACTTTGTGTACAATGAAGGT
          *** 486
  19  Q  Q  F  M  P  W  Y  K  F  D  E  N  Y  A  F  L  H  R  T  L
 540  CAGCAATTCATGCCTTGGTACAAATTCGATGAAAATTATGCATTCCTGCACCGCACGCTA
            *** 549
  39  K  E  I  L  R  Y  L  M  D  P  D  T  F  T  F  N  F  N  N  D
 600  AAGGAGATTCTCAGATACCTGATGGATCCAGACACATTCACTTTCAACTTTAATAATGAC
                       *** 621
  59  P  L  V  L  R  R  R  Q  T  Y  L  C  Y  E  V  E  R  L  D  N
 660  CCTTTGGTCCTTCGACGGCGCCAGACCTACTTGTGCTATGAGGTGGAGCGCCTGGACAAT
                * 673
  79  G  T  W  V  L  M  D  Q  H  M  G  F  L  C  N  E  A  K  N  L
 720  GGCACCTGGGTCCTGATGGACCAGCACATGGGCTTTCTATGCAACGAGGCTAAGAATCTT
                     *** 735     *** 747
  99  L  C  G  F  Y  G  R  H  A  E  L  R  F  L  D  L  V  P  S  L
 780  CTCTGTGGCTTTTACGGCCGCCATGCGGAGCTGCGCTTCTTGGACCTGGTTCCTTCTTTG

 119  Q  L  D  P  A  Q  I  Y  R  V  T  W  F  I  S  W  S  P  C  F
 840  CAGTTGGACCCGGCCCAGATCTACAGGGTCACTTGGTTCATCTCCTGGAGCCCCTGCTTC

 139  S  W  G  C  A  G  E  V  R  A  F  L  Q  E  N  T  H  V  R  L
 900  TCCTGGGGCTGTGCCGGGGAAGTGCGTGCGTTCCTTCAGGAGAACACACACGTGAGACTG

 159  R  I  F  A  A  R  I  Y  D  Y  D  P  L  Y  K  E  A  L  Q  M
 960  CGCATCTTCGCTGCCCGCATCTATGATTACGACCCCCTATATAAGGAGGCGCTGCAAATG

 179  L  R  D  A  G  A  Q  V  S  I  M  T  Y  D  E  F  E  Y  C  W
1020  CTGCGGGATGCTGGGGCCCAAGTCTCCATCATGACCTACGATGAGTTTGAGTACTGCTGG

 199  D  T  F  V  Y  R  Q  G  C  P  F  Q  P  W  D  G  L  E  E  H
1080  GACACCTTTGTGTACCGCCAGGGATGTCCCTTCCAGCCCTGGGATGGACTAGAGGAGCAC

 219  S  Q  A  L  S  G  R  L  R  A  I  L  Q  N  Q  G  N  stop
1140  AGCCAAGCCCTGAGTGGGAGGCTGCGGGCCATTCTCCAGAATCAGGGAAACTGAAGGATG
1200  GGCCTCAGTCTCTAAGGAAGGCAGAGACCTGGGTTGAGCAGCAGAATAAAAGATCTTCTT
1260  CCAAGAAATGCAAACGAGCCGTTCACCACCATCTCCAGCTGCTCACAGACACCAGCAAAG
1320  CAATGTGCTCCTGATCAAGTAGATTTTTTAAAAATCAGAGTCAATTAATTTTAATTGAAA
1380  ATTTCTCTTATGTTCCAAGTGTACAAGAGTAAGATTATGCTCAATATTCCCAGAATAGTT
1440  TTCAATGTATTAATGAAGTGATTAATTGGCTCCATATTTAGACTAATAAAACATTAAGAA
1501  TCTTCCATAATTGTTTCCACAAACACT polyA 1526
```

Figure 4. Nucleotide and deduced amino acid sequence of the phorbolin-1-related cDNA. Nucleotide and corresponding amino acid sequence of the phorbolin-1-related cDNA clone. The cDNA, which contains 1526 bp, codes for a protein having 235 amino acids starting at nucleotide position 486 and ending with a termination codon at position 1191. The in-frame upstream stop codon at position 372 is underlined and in italics. ORF start codons are numbered with their corresponding nucleotide positions and are marked with asterisks. uORF AUG codons are underlined. Accession number U61084.

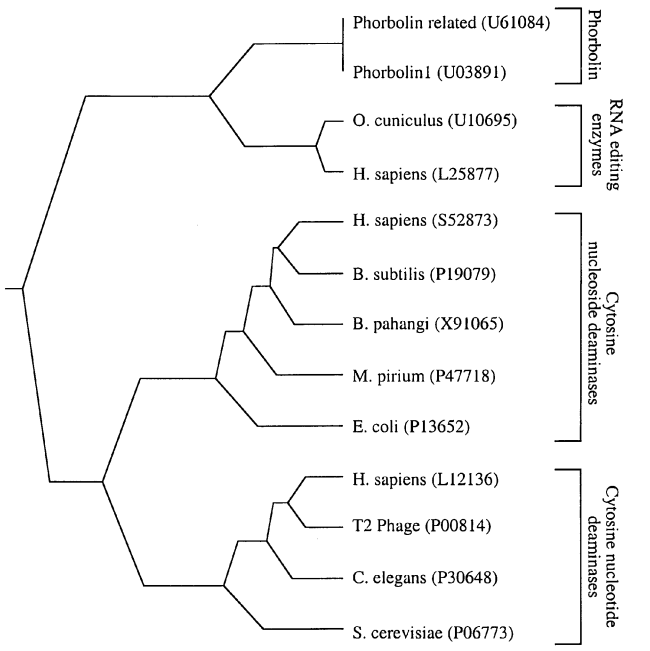

Figure 5. Phylogenetic analysis of the active sites of cytidine deaminases and the Phorbolin-1. The active site domain containing the zinc coordinating and proton donor residues were aligned as previously described based on the crystal structure of the *E. coli* cytidine deaminase (Betts *et al*, 1994; Anant *et al*, 1997). The distance matrix between the various cytidine deaminases was calculated using the PROTDIST program and used to generate a tree by the KITSCH program.

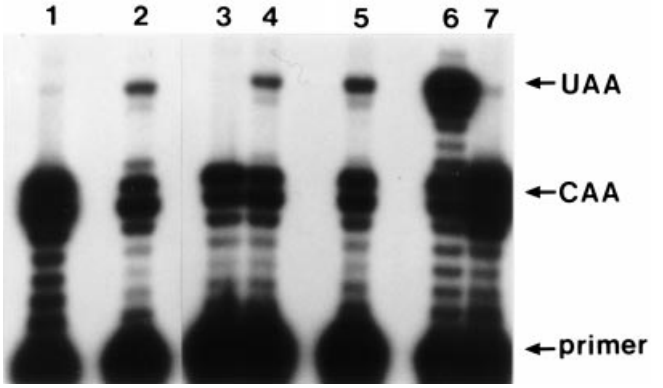

Figure 6. *In vitro* RNA editing assay. A 470 nucleotide apoB RNA was incubated with 10 μg of chicken enterocyte S-100 extract and *in vitro* transcribed/translated proteins, followed by primer extension using a radiolabeled primer (Anant *et al*, 1995). *Lane* 1, *in vitro* transcription/translation extract without plasmid DNA; *lane* 2, *in vitro* synthesized apobec-1; *lane* 3, *in vitro* synthesized phorbolin-1; *lane* 4, *in vitro* synthesized phorbolin-1 plus apobec-1; *lane* 5, *in vitro* synthesized PA-FABP (Madsen *et al*, 1992) plus apobec-1; *lane* 6, bacterially expressed and purified GST/apobec-1; *lane* 7, negative control, apoB RNA alone. This is a representative of two independent assays.

All three of these residues were shown to be necessary for apoB RNA editing activity and were, in addition, required for RNA-binding activity of recombinant apobec-1 (Anant *et al*, 1995; MacGinnitie *et al*, 1995; Navaratnam *et al*, 1995).

The similarity of the phorbolins to apobec-1 was further substantiated by a PHYLIP analysis, using the distance analysis program PROTDIST. The analysis of the active site of the phorbolins with that of apobec-1 and with other cytosine nucleoside and cytosine nucleotide deaminases also suggested that the phorbolins form a separate cluster with the apobec-1 sequences that is distinct from the other deaminases (**Fig 5**). Previous analyses have shown that the RNA-editing enzymes form a separate subgroup, and association of the phorbolins with this group suggests that the phorbolins may have evolved in order to edit a currently unknown RNA. This apparent homology between apobec-1 and the phorbolins raised the possibility that the phorbolins may compete or interact with apobec-1 in the editing of apoB RNA (**Fig 6**). Accordingly, we used preparations of phorbolin-1, synthesized in the TNT system, in an *in vitro* apoB RNA editing reaction, followed by primer extension analysis and SDS–PAGE of the edited products. Several controls were included to validate the use of this material (**Fig 6**). A representative assay is shown in **Fig 6**, which illustrates that phorbolin-1 demonstrates neither complementation of apobec-1 (*lane 3*) nor competition with apobec-1 in a standardized *in vitro* apoB RNA editing assay (*lane 4*). In addition, as alluded to earlier, previous experiments demonstrated that recombinant apobec-1 has authentic cytidine deaminase activity on a monomeric nucleoside substrate (MacGinnitie *et al*, 1995). Despite the observation that phorbolins contain the conserved catalytic domain found in other cytidine deaminases, direct experimental examination revealed no detectable cytidine deaminase activity using bacterially expressed phorbolin-1-related protein (data not shown). It remains formally possible that, as the assay used a chimeric GST fusion protein,

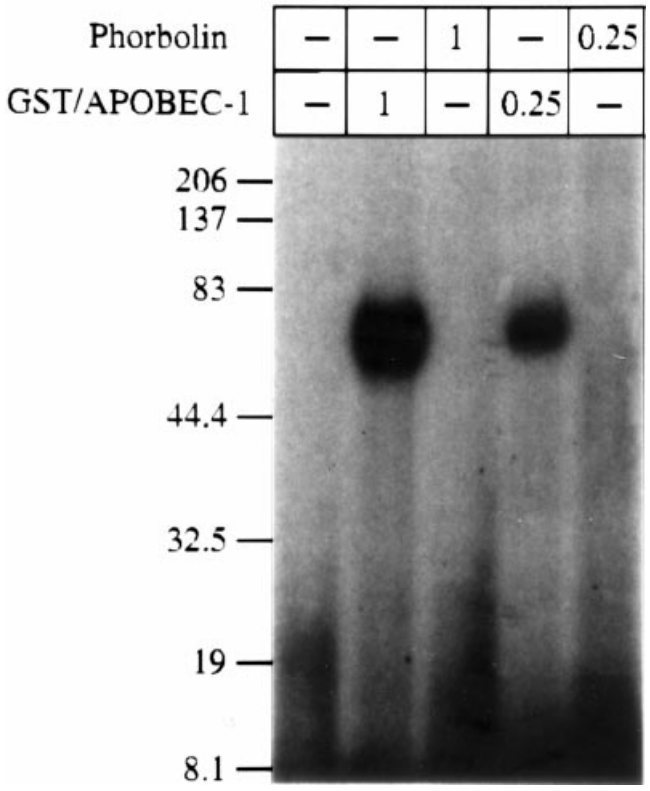

Figure 7. Phorbolin-1-related does not bind to an apoB RNA template. Ultraviolet cross-linking was performed using a radiolabeled 105 nucleotide rat apoB RNA template in the presence of 0.25 or 1 μg of recombinant GST/APOBEC-1 or GST/phorbolin-1. After digestion with RNAse T1, the samples were size fractionated in to a 10% SDS–PAGE gel, dried, and autoradiographed. Molecular weight markers are shown to the left. This is a representative of two independent assays.

conformational changes resulting from the GST moiety interfered with any potential cytidine deaminase activity. Although we have no direct evidence for this, further experiments will need to be conducted using cleaved protein. Another important activity of apobec-1 concerns its ability to bind RNA substrates, particularly those rich in A + U sequences (Anant *et al*, 1995). We examined the ability of a GST-phorbolin-1-related fusion protein to bind to a candidate AU-rich template, namely, a [^{32}P]-labeled rat apoB RNA sequence made up of 105 nucleotides that flanked the edited base. The protein–RNA complexes were ultraviolet cross-linked and visualized by SDS–PAGE. As shown in **Fig 7**, there was no evidence for RNA binding of either 0.25 or 1 μg of GST-phorbolin-1-related protein, under conditions in which the same quantities of GST-apobec-1 protein demonstrated a distinct protein–RNA complex. These findings suggest that the phorbolin-1-related protein does not function as an apoB RNA binding protein, although it should be emphasized that the possibility of its binding to other RNA templates cannot be excluded.

Taken together, the results demonstrate that the phorbolins do not manifest any of the functional properties previously ascribed to apobec-1 (Anant *et al*, 1995; MacGinnitie *et al*, 1995). Consequently, it must be concluded that, despite the apparent similarity of the primary sequences, no functional significance can be ascribed at present to the expression of phorbolins that in any way duplicates or complements the activity of apobec-1.

Chromosome mapping Using the fluorescence *in situ* hybridization technique (Tommerup and Vissing, 1995), we mapped the phorbolin genes to chromosome 22q (not shown). To refine the chromosomal localization of the phorbolin genes, we used two chromosome 22-specific cosmid libraries (LL22NC01 and LL22NC03) constructed at the Lawrence Livermore National Laboratory, CA, and permanently arranged in microtiter-format (Xie *et al*, 1993). The full list of positive cosmids is available from the authors upon request. The phorbolin cDNA probes were hybridized to high-density cosmid filters prepared from the libraries, and a unique set of 26 positive cosmid clones was identified. None of the positive cosmids had been previously recognized upon screening the libraries with a large number of various genomic and cDNA probes specific for human chromosome 22. The phorbolin-1-related probe identified five cosmids, none of which were identified by the phorbolin-1 cDNA probe. These results indicate that the phorbolins genes are located close to each other in a cluster. The exact position of the phorbolin gene cluster on chromosome 22 was finally resolved by the hybridization of one of the phorbolin-positive cosmids to the high-density filter that contained YAC clones arranged in a contig spanning the entire long arm of chromosome 22 (Collins *et al*, 1995). These results show that the phorbolin genes are localized close to the markers D22S292E and D22S314E, which are positioned at 22q13 immediately centromeric to the PDGFB gene (c-*sis* proto-oncogene) (Collins *et al*, 1995).

DISCUSSION

There are at least two main features that point towards phorbolins-1 and -2 as putative players in the pathophysiology of psoriasis. First, these proteins are highly upregulated in the psoriatic plaques (Rasmussen and Celis, 1993), and second, their synthesis is modulated by only a few effectors (Rasmussen and Celis, 1993) that, so far, include PMA, an activator of PKC, and fetal bovine serum, which is known to induce abnormal keratinocyte differentiation, a characteristic of psoriasis (Rasmussen and Celis, 1993). Second messengers (Bt_2cAMP, Bt_2cGMP) as well as other effectors (basic fibroblast growth factor, transforming growth factor-α, tumor necrosis factors-α and -β, IL-1α and IL-1β, IL-2, IL-3, IL-6, IL-7, IL-8, and interferons α and γ) tested so far have failed to deregulate the synthesis of these proteins, lending support to the contention that the abnormal expression of the phorbolins in psoriasis may be linked to an altered PKC signal transduction pathway (Nishizuka, 1992). This postulate is in line with studies from other laboratories that have shown that psoriatic lesions contain elevated levels of phosphoinositide phospholipase C activity and 1,2-diacylglycerol as compared with normal skin (Fisher *et al*, 1990), and that glycosylphosphatidylinositol-anchored membrane proteins are constitutively downregulated in psoriatic skin, perhaps as a result of the increased phosphoinositide phospholipase C activity (Venneker *et al*, 1994). Furthermore, there is evidence that calcium-dependent PKC activity is lower in psoriatic lesions than in normal skin and that is most likely due to a downregulation of the PKC-βII variant (Horn *et al*, 1987). These observations have led to the notion that the PKC pathway is altered in psoriatic skin, and it has been suggested that PKC inhibitors may have therapeutic value (Gupta *et al*, 1988; Hegemann *et al*, 1992a,b).

The potential differential usage of AUG codons by the phorbolins is interesting as in some cases this process has been shown to yield products with different functional characteristics (Nishizuka, 1992; Geballe and Morris, 1994). For example, a single transcript from the C/EBPβ gene, encodes the liver-enriched transcriptional activator LAP and the alternative product LIP, which initiates at the third AUG codon and functions as a transcriptional repressor. The relative abundance of the two forms determines the transcriptional effect, which, in turn, is developmentally regulated (Descombes and Schibler, 1991). In addition, both the selection of AUG start codons and the tissue-specific expression of genes have been shown to be regulated by uORF. Thus, the presence of a small uORF in the C/EBPα mRNA has been shown to play a crucial part in the leaky scanning mechanism of ribosomes causing a fraction of them to ignore the first C/EBPα codon (Calkhoven *et al*, 1994). Furthermore, the tissue-specific expression of the retinoic receptor-β2 (RAR β2) gene in mouse embryos is regulated at the translational level by short uORF and there is experimental evidence indicating

that the uORF4 amino acid coding sequence is important for the inhibitory effect on the translation of the downstream major ORF (Reynolds *et al*, 1996). Further analysis of psoriatic skin is necessary to establish whether the phorbolin cDNAs are translated using differential start codons.

The presence of multiple uORF in the phorbolin-1-related cDNA reveals a complex mRNA structure that may be under translational control, both with respect to translational activity and to selection of AUG start codons. Furthermore, the presence of an in-frame stop codon at position 673 raises the possibility that the mRNA may be edited, thereby allowing the synthesis of N-terminal truncated variants that may have altered biochemical activities relative to variants translated from more upstream AUG start codons. Phorbolin-1-related mRNAs having a stop codon at position 673 will most likely be subject to translational regulation, as this mRNA has 18 uORF AUG codons that will probably suppress translation from the AUG codon at position 735. The role of the uORF as well as the potential editing of the phorbolin-1-related mRNA will require additional work and will be the subject of future studies. It should be noted that the presence of uORF is strongly biased towards products of proto-oncogenes, transcriptional factors, genes encoding growth factors, and cell-surface receptors (Kozak, 1989; Geballe and Morris, 1994), lending weight to the notion that the phorbolins may be involved in an important regulatory process.

Despite the apparent sequence similarity to apobec-1, particularly within the region encompassing the catalytic domain, including several residues demonstrated to be required for enzymatic activity as well as RNA binding, no functional activity of phorbolins could be demonstrated, at least with the available assays. It remains a possibility that the phorbolins could function in the context of other RNA targets, such as those suggested for neurofibromatosis (NF) 1, but this remains purely speculative at present. To date, two other transcripts have been shown to undergo base modification, possibly affecting tumor suppressor activity (Ashkenas, 1997). These include the NF type I gene product, where a cytidine is converted into uridine (Skuse *et al*, 1996; Cappione *et al*, 1997), and the Wilms' tumor susceptibility (WT1) gene product, where a uridine is converted into cytidine (Sharma *et al*, 1994). NF1 editing is believed to occur in a fashion similar to that observed in apoB (Skuse *et al*, 1996; Cappione *et al*, 1997). Interestingly, the level of the NF1 protein is reduced in psoriatic skin, as detected by indirect immunofluorescence, and as a result, it has been suggested that this protein may play a part in the disease (Peltonen *et al*, 1995). Studies are currently underway to determine: (i) if abnormal levels of edited NF1 transcript are present in psoriatic skin, and (ii) the potential involvement of the phorbolins in this process.

Note added in proof: Recently two genomic clones from chromosome 22 have been sequenced and been found to contain sequences homologous to the phorbolin cDNA described here (acc. no. AL022318 and AL031846).

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REFERENCES

Anant S, MacGinnitie AJ, Davidson NO: Apobec-1, the catalytic subunit of the mammalian apolipoprotein B mRNA editing enzyme, is a novel RNA-binding protein. *J Biol Chem* 270:14762–14767, 1995

Anant S, Martin SA, Yu H, MacGinnitie AJ, Devaney E, Davidson NO: A cytidine deaminase expressed in the post-infective L3 stage of the filarial nematode, *Brugia pahangi*, has a novel RNA-binding activity. *Mol Biochem Parasitol* 88:105–114, 1997

Ashkenas J: Gene regulation by mRNA editing. *Am J Hum Genet* 60:278–283, 1997

Barker JN: The immunopathology of psoriasis. *Baillières Clin Rheumatol* 8:429–438, 1994

Barker JN: Adhesion molecules in cutaneous inflammation. *CIBA Found Symp* 189:91–101; 1995

Betts L, Xiang S, Short SA, Wolfenden R, Carter CWJ: Cytidine deaminase. The 2.3 A crystal structure of an enzyme: transition-state analog complex. *J Mol Biol* 235:635–656, 1994

Bhattacharya S, Navaratnam N, Morrison JR, Scott J, Taylor WR: Cytosine nucleoside/nucleotide deaminases and apolipoprotein B mRNA editing. *Trends Biochem Sci* 19:105–106, 1994

Bravo R, Celis JE: Up-dated catalogue of HeLa cell proteins: percentages and characteristics of the major cell polypeptides labeled with a mixture of 16 [^{14}C]-labeled amino acids. *Clin Chem* 28:766–781, 1982

Calkhoven CF, Bouwman PR, Snippe L, Ab G: Translation start site multiplicity of the CCAAT/enhancer binding protein alpha mRNA is dictated by a small 5′ open reading frame. *Nucleic Acids Res* 22:5540–5547, 1994

Cappione AJ, French BL, Skuse GR: A potential role for NF1 mRNA editing in the pathogenesis of NF1 tumors. *Am J Hum Genet* 60:305–312, 1997

Celis JE, Basse B, Lauridsen JB. Determination of antibody specificity by Western blotting and immunoprecipitation. In: Celis JE (ed.). *Cell Biology. A Laboratory Handbook*, Vol. 2. San Diego: Academic Press, 1994a: pp 305–321

Celis JE, Ratz G, Basse B, Lauridsen JB, Celis A. high-resolution two dimensional gel electrophoresis of proteins: isoelectric focusing and nonequilibrium pH gradient electrophoresis (NEPHGE). In: Celis JE (ed.). *Cell Biology: a Laboratory Handbook*, Vol. 3. San Diego: Academic Press, 1994b: pp 222–230

Celis JE, Gromov P, Ostergaard M, *et al*: Human 2-D PAGE databases for proteome analysis in health and disease: http://biobase.dk/cgi-bin/celis. *FEBS Lett* 398:129–134, 1996

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294–5299, 1979

Christensen JH, Hansen PK, Lillelund O, Thøgersen HC: Sequence-specific binding of the N-terminal three-finger fragment of *Xenopus* transcription factor IIIA to the internal control region of a 5S RNA gene. *FEBS Lett* 281:181–184, 1991

Christophers E: Psoriasis: mechanisms and entry points for possible therapeutic interventions. *Australas J Dermatol* 37 (Suppl. 1):S4–S6, 1996

Collins JE, Cole CG, Smink LJ, *et al*: A high-density YAC contig map of human chromosome 22. *Nature* 377:367–379, 1995

Descombes P, Schibler U: A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* 67:569–579, 1991

Elder JT, Tavakkol A, Klein SB, Zeigler ME, Wicha M, Voorhees JJ: Protooncogene expression in normal and psoriatic skin. *J Invest Dermatol* 94:19–25, 1990

Felsenstein J: Phylogenies from molecular sequences: inference and reliability. *Annu Rev Genet* 22:521–565, 1988

Fisher GJ, Talwar HS, Baldassare JJ, Henderson PA, Voorhees JJ: Increased phospholipase C-catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate and 1,2-sn-diacylglycerol content in psoriatic involved compared to uninvolved and normal epidermis. *J Invest Dermatol* 95:428–435, 1990

Fisher GJ, Tavakkol A, Leach K, *et al*: Differential expression of protein kinase C isoenzymes in normal and psoriatic adult human skin: reduced expression of protein kinase C-beta II in psoriasis. *J Invest Dermatol* 101:553–559, 1993

Geballe AP, Morris DR: Initiation codons within 5′-leaders of mRNAs as regulators of translation. *Trends Biochem Sci* 19:159–164, 1994

Giannoni F, Bonen DK, Funahashi T, Hadjiagapiou C, Burant CF, Davidson NO: Complementation of apolipoprotein B mRNA editing by human liver accompanied by secretion of apolipoprotein B48. *J Biol Chem* 269:5932–5936, 1994

Gubler U, Hoffman BJ: A simple and very efficient method for generating cDNA libraries. *Gene* 25:263–269, 1983

Gupta AK, Fisher GJ, Elder JT, Nickoloff BJ, Voorhees JJ: Sphingosine inhibits phorbol ester-induced inflammation, ornithine decarboxylase activity, and activation of protein kinase C in mouse skin. *J Invest Dermatol* 91:486–491, 1988

Hegemann L, van Bonnekoh B, Rooijen LA, Mahrle G: Anti-proliferative effects of protein kinase C inhibitors in human keratinocytes. *J Dermatol Sci* 4:18–25, 1992a

Hegemann L, van Fruchtmann R, Rooijen LA, Muller-Peddinghaus R, Mahrle G: The antipsoriatic drug, anthralin, inhibits protein kinase C—implications for its mechanism of action. *Arch Dermatol Res* 284:179–183, 1992b

Hochuli E, Dobeli H, Schacher A: New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J Chromatogr* 411:177–184, 1987

Honoré B, Madsen P: The tetramethylammonium chloride (TMAC) method for screening cDNA libraries with highly degenerate oligonucleotide probes obtained by reverse translation of amino acid sequences. *Methods Mol Biol* 69:139–146, 1997

Horn F, Marks F, Fisher GJ, Marcelo CL, Voorhees JJ: Decreased protein kinase C activity in psoriatic versus normal epidermis. *J Invest Dermatol* 88:220–222, 1987

Kozak M: The scanning model for translation: an update. *J Cell Biol* 108:229–241, 1989

Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970

MacGinnitie AJ, Anant S, Davidson NO: Mutagenesis of apobec-1, the catalytic subunit of the mammalian apolipoprotein B mRNA editing enzyme, reveals

distinct domains that mediate cytosine nucleoside deaminase, RNA binding, and RNA editing activity. *J Biol Chem* 270:14768–14775, 1995

Madsen P, Rasmussen HH, Leffers H, *et al*: Molecular cloning, occurrence, and expression of a novel partially secreted protein 'psoriasin' that is highly up-regulated in psoriatic skin. *J Invest Dermatol* 97:701–712, 1991

Madsen P, Rasmussen HH, Leffers H, Honoré B, Celis JE: Molecular cloning and expression of a novel keratinocyte protein (psoriasis-associated fatty acid-binding protein [PA-FABP]) that is highly up-regulated in psoriatic skin and that shares similarity to fatty acid-binding proteins. *J Invest Dermatol* 99:299–305, 1992

Madsen P, Gromov P, Celis JE: Expression of cDNA clones by coupled *in vitro* transcription/translation and transfection into COS-1 cells: protein mapping in two-dimensional gels. *Electrophoresis* 16:2258–2261, 1995

Navaratnam N, Bhattacharya S, Fujino T, Patel D, Jarmuz AL, Scott J: Evolutionary origins of apoB mRNA editing: catalysis by a cytidine deaminase that has acquired a novel RNA-binding motif at its active site. *Cell* 81:187–195, 1995

Nishizuka Y: Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258:607–614, 1992

Norris DA, Travers JB, Leung DY: Lymphocyte activation in the pathogenesis of psoriasis. *J Invest Dermatol* 109:1–4, 1997

O'Farrell PH: High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250:4007–4021, 1975

Peltonen J, Karvonen SL, Yla-Outinen H, Hirvonen O, Karvonen J: Lesional psoriatic epidermis displays reduced neurofibromin immunoreactivity. *J Invest Dermatol* 105:664–667, 1995

Rasmussen HH, Celis JE: Evidence for an altered protein kinase C (PKC) signaling pathway in psoriasis. *J Invest Dermatol* 101:560–566, 1993

Reynolds K, Zimmer AM, Zimmer A: Regulation of RAR beta 2 mRNA expression: evidence for an inhibitory peptide encoded in the 5′-untranslated region. *J Cell Biol* 134:827–835, 1996

Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463–5467, 1977

Sharma PM, Bowman M, Madden SL, Rauscher FJ, Sukumar S: RNA editing in the Wilms' tumor susceptibility gene, WT1. *Genes Dev* 8:720–731, 1994

Skuse GR, Cappione AJ, Sowden M, Metheny LJ, Smith HC: The neurofibromatosis type I messenger RNA undergoes base-modification RNA editing. *Nucleic Acids Res* 24:478–485, 1996

Teng B, Burant CF, Davidson NO: Molecular cloning of an apolipoprotein B messenger RNA editing protein. *Science* 260:1816–1819, 1993

Tommerup N, Vissing H: Isolation and fine mapping of 16 novel human zinc finger-encoding cDNAs identify putative candidate genes for developmental and malignant disorders. *Genomics* 27:259–264, 1995

Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354, 1979

Valdimarsson H, Baker BS, Jonsdottir I, Powles A, Fry L: Psoriasis: a T-cell-mediated autoimmune disease induced by streptococcal superantigens? *Immunol Today* 16:145–149, 1995

Rasmussen HH, Van Damme J, Bauw M, Puype M, Gesser B, Celis JE, Vanderkerckhove J. Protein-electroblotting and microsequencing in establishing integrated human protein databases. In: Jörnvall H, Höög JO, Gustavsson AM (eds). *Methods in Protein Sequence Analysis*. Basel: Birkhäuser, 1991:pp 103–141

Vanderkerckhove J, Rasmussen HH. Internal amino acid sequencing of proteins recovered from one- or two dimensional gels. In: Celis JE (ed.) *Cell Biology. A Laboratory Handbook*, Vol. 3. San Diego: Academic Press, 1994:pp 359–368

Venneker GT, Das PK, van Meinardi MM, Marle J, Veen HA, Bos JD, Asghar SS: Glycosylphosphatidylinositol (GPI) -anchored membrane proteins are constitutively down-regulated in psoriatic skin. *J Pathol* 172:189–197, 1994

Wiedow O, Young JA, Davison MD, Christophers E: Antileukoprotease in psoriatic scales. *J Invest Dermatol* 101:305–309, 1993

Wright NA, Camplejohn RS: *Psoriasis: Cell proliferation*. Edinburgh: Churchill Livingstone, 1985,

Xie YG, Han FY, Peyrard M, *et al*: Cloning of a novel, anonymous gene from a megabase-range YAC and cosmid contig in the neurofibromatosis type 2/meningioma region on human chromosome 22q12. *Hum Mol Genet* 2:1361–1368, 1993